Invariant Natural Killer T Cells and Immunotherapy of Cancer

Molling, J.W.

2008

document version
Publisher's PDF, also known as Version of record

Chapter 8

Invariant Natural Killer T cells and immunotherapy of cancer

Johan W. Molling, María Moreno, Hans J.J. van der Vliet, Alfons J.M. van den Eertwegh, Rik J. Scheper, B. Mary E. von Blomberg and Hetty J. Bontkes

Cancer Center Amsterdam, VUMC Institute for Cancer and Immunology (V-ICI), Division of Immune Therapy, Departments of Medical Oncology and Pathology

Submitted for publication

Abstract

Invariant CD1d restricted natural killer T (iNKT) cells are a regulatory cells that express a canonical TCR-Vα-chain (Vα24.Jα18 in humans and Vα14.Jα18 in mice) which recognizes glycolipid antigens presented by the monomorphic CD1d molecule. They can secrete of a wide variety of both pro-inflammatory and anti-inflammatory cytokines very swiftly upon their activation. And in pre-clinical models they have the capacity to enhance host immunity to microorganisms and cancers as well as to prevent autoimmunity.
Direct evidence suggesting the significance of iNKT cells in human cancer has been ambiguous. The (pre)-clinical findings reviewed here, provide further evidence for a distinct contribution of iNKT cells to natural anti-tumor immune responses in humans. Furthermore, clinical phase 1 studies that are discussed here have revealed that the infusion of cancer patients with soluble TCR ligand for iNKT cells, with ligand loaded dendritic cells, or with cultured iNKT cells is well tolerated. We thus underscore the potential of iNKT cell based immunotherapy in conjunction with established modalities such as surgery and radiotherapy, as an immunotherapeutic adjuvant therapy against carcinomas.

Introduction

The complex relationship between the immune system and human cancer has been thoroughly investigated for decades. An effective anti-tumor immune response appears to be important to eradicate malignant cells from the body. This is illustrated by the observations in immunocompromised patients, who have a higher incidence of tumors (reviewed in [1]). The microenvironment of progressing tumors that have evaded eradication by effector cells is similar to sites of chronic inflammation. This microenvironment is characterized by angiogenic and tumor growth factors which promote tumor growth and the induction of immunosuppressive cells (reviewed in [2-4]). An immune response mediated by effector cells without the development of a state of chronic inflammation is vital for a successful anti-tumor immune response. Here we provide evidence that a relatively recently discovered T-cell subset with immune controlling capacity holds promise in this respect.
Invariant CD1d restricted natural killer T (iNKT) cells can express NK receptors and express a canonical TCR-Vα-chain (Vα24.Jα18 in humans, preferentially paired with Vβ11; Vα14.Jα18 in mice, paired with Vβ2, Vβ7 or Vβ8.2) recognizing glycolipid antigens presented by the monomorphic CD1d molecule [5;6]. The glycolipid α-galactosylceramide (αGalCer) originally isolated from the marine sponge *Agelas mauritianus* is a strong synthetic ligand for iNKT cells, which induces iNKT cell proliferation and activation. More importantly, iNKT cells can readily secrete high levels of a broad range of both pro- (Th1) and anti- (Th2) inflammatory cytokines upon activation [5;7]. Glycolipids derived from microbial pathogens and a (currently disputed [8;9]) endogenous ligand, the lysosomal glycosphingolipid isoglobotrihexosylceramide (iGb3), can also trigger iNKT cell activation *in vitro*.[10-14]. The swift and wide-ranging *in vitro* cytokine release reveals the main function of iNKT cells, namely the capacity to enhance immune responses against microorganisms and cancer as well as to prevent autoimmunity by acting on down-stream immune cells (Reviewed in [15]). Human and mouse iNKT cells can either be $CD4^+$ or $CD4^-CD8^-$ (double negative (DN)) and in humans a small proportion can express CD8. Direct *ex vivo* analyses suggested that CD4+ iNKT cells produce both Th1 cytokines (e.g GM-CSF, IFNγ and TNF-α) and Th2 cytokines (e.g. IL-4 and IL-13), whereas the DN and CD8+ iNKT cell subsets primarily produce Th1 cytokines [16-19]. These differences in iNKT cell subsets are the most likely explanation for their dichotomous regulatory nature. Lin et al reported that in their model *in vitro* expanded $CD4^+$ iNKT cells were more capable than $CD4^-$ iNKT cells of activating T, B and NK cells in response to αGalCer, probably because they very potently released IFN-γ and IL-4. [20]. On the other hand, Osada et al demonstrated that *in vitro* expanded $CD4^+$ (but not $CD4^-$) iNKT cells could inhibit the *in vitro* induction of MART-1 specific memory $CD8^+$ T cells in the

presence of αGalCer. This suppression appeared to be associated with an enhanced secretion of the type 2 cytokines IL-4, IL-5 and IL-10 [21]. The different methods used for the expansion of iNKT cells, the use of the type 1 skewing cytokine IL-15 [22] in particular, which was used in the latter study, may explain these differences. Alternatively, the different read-outs (*in vitro* survival/ expansion of antigen specific T cells vs. up-regulation of activation markers) might explain the opposing conclusions drawn from these studies. In addition, the culture methods used to generate *in vitro* iNKT cells described above could influence their effect on other immune cells. For example, we were able to enhance the *in vitro* capacity of iNKT cells to augment tumor associated antigen specific CD8 T cell expansion (Bontkes HJ, *unpublished results*) and NK cell mediated killing of tumor cell lines (Moreno M, *unpublished results*) only when we transfected dendritic cells (DC) with IL-12 mRNA before pulsing them with αGalCer.

An important physiological role for iNKT cells was shown in the immuno-surveillance of cancers. Mice deficient in iNKT cells (Jα18$^{-/-}$ mice) were more susceptible to chemically (methylcholanthrene (MCA)) induced sarcomas, while protection could be restored by adoptive transfer of iNKT cells isolated from Wild-type animals. Protection depended on CD1d, IFN-γ production by iNKT cells, and NK and CD8 T-cell function [23]. The capacity of *in vivo* activated iNKT cells to enhance protection against experimental tumors has been studied extensively. Depending on the model, resident iNKT cells can augment innate as well as adaptive anti-tumor immune responses. Several groups demonstrated that systemic injection of αGalCer or αGalCer loaded DC activates iNKT cells, leading to the inhibition of metastasis formation predominantly via the down-stream activation of NK cells [24-27].

Studies combining i.v. free αGalCer treatment with protein vaccination demonstrated that αGalCer acts as an adjuvant for the induction of antigen specific CD4$^+$ and CD8$^+$ T cell mediated immunity [28;29]. Furthermore, a single i.v. injection of dying hematologic tumor cells together with αGalCer induced long lasting protective immunity, depending on conventional CD4$^+$ and CD8$^+$ T cells. In this model resident αGalCer activated iNKT cells enhanced the maturation of DC that subsequently were more efficient in cross priming CD4$^+$ and CD8$^+$ T cells [30].

These intriguing findings from pre-clinical studies prompted several groups to study iNKT cell numbers and function in cancer patients and to perform clinical phase I studies in these patients on the *in vivo* activation of iNKT cells through administration of αGalCer, αGalCer pulsed DC or iNKT enriched autologous PBMC. This review will focus on the consequences of these findings for future iNKT cell mediated immunotherapy. In the first part we will provide evidence for the relevance of iNKT cells in human cancer. In the second part we will discuss the promising results that have been obtained from several clinical phase I studies on iNKT cell activation and, in particular, we will illustrate the therapeutic potential of autologous adoptive transfer of purified iNKT cell lines in patients that are severely deficient in iNKT cells.

Part I: Relevance of invariant Natural Killer T lymphocytes in human cancer

Selective decrease of iNKT cell numbers in peripheral blood of carcinoma patients

As depicted in Table I, Kawano and colleagues were the first to report a numeric defect in circulating iNKT cells in cancer, namely in melanoma patients [31]. These findings were confirmed in patients with prostate cancer [32] and lung cancer [33;34], but not in malignant myeloma [35], glioma [36] or neuroblastoma [37].
However, the latter were all small cohorts and the effects of age and gender, reported by Delarosa et al [38] and Sandberg et al [39], were not always taken into account. We therefore studied circulating iNKT cell levels in a cohort of 120 patients with various epithelial cancers (breast, colorectal, head and neck and renal cell cancer and melanoma) and 69 healthy controls using multivariate analysis and confirmed that: a) in both patients and controls, iNKT cell levels selectively decreased with age, most prominently in males and b) after correction for age and gender, cancer patients had a selective numeric iNKT cell deficiency within the circulating T cell pool [40]. iNKT cell numbers were not influenced by tumor type or disease stage, which was also reported by Motohashi et al [33] and Konishi et al [34]. Tumor de-bulking by surgery or radiotherapy did not restore circulating iNKT cell numbers, suggesting that the numeric defect was not the consequence of tumor growth. Also Crough et al investigated circulating iNKT cell numbers in a variety of carcinomas and found a significant reduction in melanoma and breast cancer, but in contrast to our findings not in colorectal cancer or renal cell carcinoma [41]. Yanagisawa et al did not find a significant reduction in colorectal carcinoma patients either (nor in esophageal, gastric, gall bladder, uterus, bile duct and pancreas cancer) [42]. However patient groups were subdivided in these two latter studies, resulting in a loss in statistical power. Crough et al also observed an age dependent reduction in iNKT cells in accordance with our observation and, interestingly, they observed that while chemotherapy did not affect iNKT cell numbers, radiotherapy would reduce these in melanoma patients [41]. We observed a similar transient effect of radiotherapy on iNKT circulating cell levels in head and neck cancer. In paired peripheral blood samples of 27 patients a significant drop in iNKT cell numbers 4 weeks after radiotherapy was observed ($p<0.01$), while 4 months after radiotherapy no significant reduction in iNKT cell numbers was observed. Of note, this effect of radiotherapy was far more dramatic on conventional T cell levels (Molling JW, *unpublished results*). Taken together these findings indicate that circulating iNKT cell levels are at most only mildly affected by the outgrowing tumor or by chemotherapy or radiotherapy.
In our cohort of carcinoma patients, there were no differences in the percentages of $CD4^+$ and $CD4^-$ cells within the iNKT cell pool of breast cancer patients (n=9) compared to age and gender matched controls. This similarity in $CD4^+$ / $CD4^-$ ratio between cancer patients and healthy controls was also observed in glioma patients (n=9) [36]. These findings suggest that there is no imbalance in iNKT cell regulatory status in cancer patients. Again, though, it should be kept in mind that this iNKT sub-analysis was performed in relatively small groups of patients.
Some controversy exists with regard to the capacity of the residual iNKT cells of cancer patients to secrete IFN-γ or to proliferate in response to αGalCer *in vitro*. It is noteworthy, in this respect, that the decline in *ex vivo* IFN-γ secretion [40] and expansion [43] of iNKT cells in response to αGalCer was reported to be age related. Age differences between patients and control subjects in the studies reviewed here, should therefore be considered. Unfortunately, this information is not always provided. Nonetheless, in all reported studies iNKT cells from cancer patients retained their capacity to produce IFN-γ *ex vivo* upon αGalCer stimulation; albeit that in some studies repeated or highly active stimulation by professional antigen presenting cells was required.
Substantial *in vitro* proliferation of iNKT cells from cancer patients was achieved in studies using αGalCer pulsed monocytes or immature monocyte derived DC (moDC) as APC

[31;36]. In contrast, iNKT cell expansion using αGalCer pulsed autologous PBMC did not lead to adequate expansion or IFN-γ secretion by iNKT cells from patients suffering from prostate cancer [32], lung cancer [34] and in a variety of carcinomas [42]. This might be explained by the use of autologous PBMC as APC which might induce *in vitro* iNKT cell anergy, since the function of APC is often impaired in cancer patients and a reduction in functional APC has been demonstrated to result in changes in the different T-cell subsets [44]. In line with this, Tahir et al demonstrated that IFN-γ secretion by iNKT cells from prostate cancer patients was restored when recombinant IL-12 was added to iNKT cell / APC co-cultures [32]. An alternative explanation was suggested in the study by Yanagisawa et al, who provided evidence that other suppressive T cells might hamper iNKT cell expansion in colorectal cancer [42]. Indeed, naturally occurring regulatory T cell (nTreg; supposedly the key regulatory cells for maintenance of tolerance against self antigens) have been demonstrated to be capable of directly inhibiting iNKT cell proliferation, cytokine secretion and cytotoxic activity via cognate interactions [45]. Furthermore, it has been well established that circulating nTreg numbers are enhanced in a broad variety of cancers [46-49] and that they can suppress anti-tumor responses (reviewed in [50]).
It is not yet clear whether *in vivo* circulating human iNKT cells are capable of killing tumor cells. Chamoto et al demonstrated that mouse $CD3^+NK1.1^+$ NKT cells first had to be stimulated *in vitro* with αGalCer pulsed DC in the presence of IL-2 to gain NK-like cytotoxic ability. In their study, resident iNKT cells exerted their anti-tumor effects by activating down-stream effector cells *in vivo* [51]. Expanded iNKT cells of cancer patients can display *in vitro* cytotoxicity against various tumor targets [31;35;36;42]. After culture with αGalCer pulsed monocytes or moDC and exogenous IL-2, iNKT cells from melanoma patients were able to kill tumor cell lines [31] as well as autologous tumor cells [35] *in vitro*. The observed cytotoxic effects were most likely perforin dependent since they were abolished by concanamycin A treatment. This is in line with our observations that *in vitro* cultured iNKT cells contain cytotoxic granules consisting of cytotoxic effector molecules such as perforin, granzyme B, and T cell intracellular antigen-1 [52-54]. However, these *ex vivo* cultured iNKT ells do not kill very efficiently and often require loading of $CD1d^+$ tumor cells with αGalCer [22;55;56]. Furthermore, the ability to directly kill tumor cells has not been established to be a physiological function of circulating iNKT cells. Hence, in our opinion, the reduced numbers of circulating iNKT cells reflects a reduced capacity to activate downstream innate and adaptive immune effector cells.
In summary, although some discrepancies have been noted regarding iNKT cell number and function in different cancers, evidence is accumulating that circulating iNKT cells of carcinoma patients are substantially reduced compared to healthy controls. Notwithstanding, residual iNKT cells in these patients still possess the capacity to proliferate, to secrete IFN-γ and to gain cytotoxic activity when properly stimulated *in vitro.* This suggests that the residual iNKT cells of carcinoma patients might still be capable of taking part in patho-physiological anti-tumor responses or after therapies aimed at their increase and activation in cancer patients. These two aspects will be discussed further in the remaining sections of this manuscript.

Peripheral blood iNKT cells in relation to clinical outcome of cancer

The reduction in circulating iNKT cells of cancer patients is indicative of their alleged importance for efficient anti-tumor immune responses to occur. However, it has not been demonstrated in the studies reviewed above, whether a reduction of circulating iNKT cells in cancer patients precedes the development of cancer and can be regarded as a risk factor. This also remains to be elucidated for the elevated levels in cancer patients of circulating nTregs, which may inhibit iNKT cell proliferation and activation. We therefore studied prospectively the relation between peripheral blood iNKT cell and nTreg frequencies and the natural course of human papillomavirus type 16 (HPV16) induced pre-invasive cervical

intraepithelial neoplasia (CIN), in 82 patients who participated in a nonintervention cohort study of women with abnormal cervix cytology (Molling JW et al*, Int. J. Cancer in press*). Persistent infection with oncogenic high-risk HPV types, of which HPV 16 is the most prevalent type, is a major risk factor for the development of severe cervical dysplasia/carcinoma in situ (CIN3) and invasive cervical carcinoma (Walboomers). Untreated severe dysplasia/carcinoma in situ (CIN3) may lead to invasive carcinoma. Circulating nTreg numbers were increased in individuals with a persistent HPV16 infection and in patients who developed a CIN 3 lesion compared to those who were initially infected but cleared the virus and patients who did not progress to CIN3. However, within the HPV 16 persistence group Treg frequencies were equally high among patients who developed a CIN3 lesion and patients who did not progress to CIN3. The number of circulating iNKT cells was not related to either a persistent infection with the virus nor with progression of cervical dysplasia towards carcinoma in situ. However, this does not rule out that iNKT cells could be involved in immune responses directed at later stage malignancies.

In a cohort of 120 carcinoma patients (various epithelial tumors) and 69 healthy controls, we observed a wide range in the size of the circulating iNKT cell pool. While the patient group as a whole was significantly deficient in iNKT cell numbers, some individuals had iNKT cell levels resembling those observed in age-matched healthy controls whereas others had very low to undetectable numbers of iNKT cells in their circulation [40]. In a prospective study, we therefore tested the hypothesis that a severe iNKT cell deficiency was related to a poor clinical outcome after radiation therapy of head and neck squamous cell carcinoma (HNSCC). Peripheral blood samples of 47 patients were stratified according to the levels of T, NK or iNKT cells at the start of therapy and clinical data were obtained during a median follow-up period of 31 months [57]. Indeed, by Cox regression analysis we confirmed that a severe quantitative iNKT cell deficiency prior to radiation therapy was a prognostic parameter, predicting shortened (disease specific) survival and the increased risk of locoregional tumor recurrence, independently of clinical T stage and age. In the group of patients with a severe iNKT cell deficiency there was a 53 % relative loss in 3 year disease specific survival, compared to patients with above average iNKT cell levels (39 vs. 92 % 3 year survival rate). Although the pre-therapy iNKT cell levels as well as T or NK cell levels were reduced in this cohort as compared to historical age and gender matched controls, no evidence was obtained for a relation between the number of peripheral blood T or NK cells and clinical outcome, indicating that the observations specifically related to circulating iNKT cells. This was in line with previous reports on HNSCC in which circulating T or NK cell levels were reduced but without prognostic value [58-60]. Reduced functionality, rather than reduced numbers of circulating or tumor infiltrating T and NK cells was related to a poor prognosis in HNSCC in several studies [61-65]. In these retrospective studies low or absent ζ chain expression, reduced proliferation, low cytotoxicity, an altered cytokine profile and increased apoptosis of CD8+ effector cells were observed. Interestingly, Reichert et al demonstrated a strong correlation between a loss of function in peripheral T cells and a loss of function in TILs in HNSCC patients. The authors suggested that these individuals would be at risk of a poor prognosis as a result of immune compromise [64]. As mentioned above, a similar observation was made in myeloma, where a marked defect in IFN-γ secretion by iNKT cells in the circulation as well as in the tumor bed of progressive, but not of non-progressive myeloma was found [35].

These combined findings raise the possibility that the level of peripheral blood iNKT cells in HNSCC patients, of which we have evidence that they can still secrete IFN-γ [40], correlates to their relative contribution to local anti-tumor responses. It would thus be relevant to study the correlation between peripheral and intra-tumor iNKT cell number and function in future cohorts. Furthermore, relating the findings from such cohorts to T and NK cell function with regard to clinical outcome is warranted to provide more insight in how circulating iNKT cells would relate to anti-tumor responses within the tumor microenvironment.

Tumor infiltrating iNKT cells in relation to clinical outcome of cancer

To make a significant contribution to local anti-tumor responses, iNKT cells would most likely have to be located in the tumor draining lymph nodes and/ or in the tumor microenvironment. Both in humans and in germ free mice iNKT cells were found to acquire an effector (memory) phenotype before birth, allowing for their distribution throughout the peripheral tissues rather than the lymph nodes [66;67]. As such, they resemble tissue-infiltrating Th1 cells and $CD8^+$ cytotoxic T lymphocytes (CTL) and indeed have a corresponding chemokine receptor expression pattern [17;68;69]. *In vitro*, iNKT cell clones migrate towards CXCR3, CXCR4 and CCR4 ligands and to a lesser extend to ligands for CCR5 and CXCR6 [68]. The *in vivo* recruitment of iNKT cells to inflamed tissues was demonstrated in mouse models, wherein the initial influx of iNKT cells was required for the formation of granulomatous lesions caused by *Mycobacterium tuberculosis* [70] or *Cryptococcus neoformans* [71]. The latter study provided evidence that the iNKT cells were attracted by the CCR2 ligand monocyte chemoattractant protein (MCP)-1 (i.e. CCL2).
In line with this, Metelitsa et al showed that those human neuroblastomas that expressed CCL2 were well infiltrated with iNKT cells. Patients with iNKT cell infiltrated tumors had a significantly prolonged long term survival, compared to patients with tumors that were not infiltrated with iNKT cells but did contain conventional T cells [37]. Interestingly, they recently reported that amplified MYCN oncogene, which is a hallmark of aggressive neuroblastoma [72], repressed CCL2 expression leading to impaired iNKT cell infiltration. This was strikingly evident in patients with bone marrow metastases of neuroblastoma, suggesting an important contribution of iNKT cells to the immune attack against these metastases [73].
In addition, Tachibana et al demonstrated that colorectal carcinomas were well infiltrated with activated $CD69^+$ TCR-V$\alpha 24^+$ T cells compared to the patients' control tissue and established by Cox regression analysis that high TCR-V$\alpha 24^+$ T cell infiltration was predictive of prolonged (disease free) survival, independently of other prognostic variables like clinical T stage [74]. Although the authors did provide indirect evidence that the TCR-V$\alpha 24^+$ T cells were indeed iNKT cells, it is not clear whether these cells infiltrated the tumor more readily than other T cells. This is relevant since a high number of tumor infiltrating T cells is anyhow related to favorable prognosis in colorectal cancer (reviewed in [75]). A preferential influx into colorectal tumors has been demonstrated for $CCR5^+$ $CXCR3^+$ T cells [76]. IFN-γ release by T cells arriving early at the invasive margin was proposed in this study to trigger local IP-10 secretion (a known CXCR3 ligand). This would facilitate the influx of Th1 cells and CTLs involved in the subsequent anti-tumor response. Thus the high expression of CCR5 and CXCR3 on iNKT cells [68], their capacity to readily release IFN-γ in carcinoma patients [33;40] and the influx of iNKT cells into colorectal carcinomas [74] strongly suggest a significant contribution of iNKT cells to the local response against colorectal cancer. In addition, the finding by Dhodapkar et al that iNKT cells in the tumor bed of progressive myeloma were defective in IFN-γ secretion, whereas those in non progressive myeloma were competent [35] is suggestive of a causal relation between loss of iNKT cell functionality and disease progression and is in support of the hypothesis above.

Proposed mechanisms behind the role of iNKT cells in anti-tumor responses

A few mechanisms via which iNKT cells might hamper the growth of malignancies will be discussed below. First, in a model of the etiology of chronic inflammatory bowel disease, it has been proposed that heat shock protein (Hsp)110 is involved in the regulation of CD1d expression in human intestinal epithelia [77;78]. In this model the constitutive expression of CD1d on intestinal epithelial cells is maintained by a steady state release of Hsp110 during cell renewal. Upon epithelial tissue damage Hsp110 release is enhanced, resulting in the constitutive up-regulation of CD1d expression and subsequent activation of resident iNKT cells to secrete Th1 cytokines, leading to chronic inflammation. In addition, the enlarged

deposits of Hsp70 in immunohistochemical stainings of atherosclerotic lesions were found to co-localize with expression of CD1d and CD1a, but not other MHC (like) molecules, on DC within these lesions [79]. Surface CD1d was also increased on keratinocytes in psoriatic lesions [80] and these lesions have been shown to express Hsp70 [81]. Furthermore, Hsp expression has been reported for human tumor cells *in vitro* and *in vivo* [82;83]. Viable tumor cells co-injected with Hsp expressing apoptotic tumor cells were more efficiently rejected by recipient mice than viable tumor cells alone [84]. It is therefore tempting to speculate that locally produced heat shock proteins at sites of (metastasized) malignant lesions, either by tumor cells or damaged epithelial cells, can trigger the activation of resident iNKT cells by enhancing CD1d mediated antigen presentation within the tumor micro-environment (Figure 1). In the light of HPV16 induced cervical carcinoma, an interesting discrimination has been made between pre-malignant CIN and invasive cervical cancer regarding Hsp expression. In a cohort of 30 patients with CIN lesions (6 moderate, 11 severe and 13 carcinoma in situ) and 20 patients with invasive cervical cancer, Hsp70 expression was significantly elevated in the latter group and was associated with tumor cell proliferation (i.e. Ki-67 staining) [85]. Although CD1d expression has not been studied in cervical cancers in relation to Hsp expression, these data suggest that iNKT cells might indeed come into play in anti-tumor immune responses at a relatively late stage of tumor development.

Second, several studies have demonstrated the capacity of iNKT cells to stimulate DC activation and maturation [28-30;86;87]. In the setting proposed above, iNKT cells could enhance DC maturation within the invasive margin resulting in improved migration of DC towards the tumor draining lymph nodes and thus in more effective priming of additional naive $CD4^+$ and $CD8^+$ T cells with tumor derived antigens [88-91] (Figure 2A).

Third, tumor cells in blood or lymphatic circulation are particularly sensitive to NK mediated lysis which is considered to be most effective in the presence of anti-tumor immunoglobulins via antibody-dependent cellular cytotoxicity (ADCC) (reviewed in [2]). For efficient locoregional tumor control to occur (which was associated with circulating iNKT cells levels in HNSCC [57]) the local vasculature would obviously be a critical location for the immune system to attack metastatic cells released by the primary tumor. Although *ex vivo* cultured iNKT cells were reported to kill tumor targets *in vitro*, they did not kill very efficiently and often required loading of $CD1d^+$ tumor cells with αGalCer [22;55;56]. Furthermore, the ability to kill tumor cells has not been established as a physiological function of circulating iNKT cells. In mice, iNKT cells had to be cultured *in vitro* to acquire direct cytotoxic capacity whereas *in vivo* they mainly instructed NK cells to eradicate i.v. injected tumor cells [51]. In our opinion it is therefore unlikely that peripheral iNKT cells of cancer patients are capable of directly eradicating metastasizing tumor cells. However, equivalent to their murine counterpart [92], human iNKT cells can activate NK cells as well as B cells [55;93] and might thus be able to promote a tumoricidal environment within the vicinity of the tumor (Figure 2B).

Fourth, angiogenesis and lymphangiogenesis are of vital importance for a malignant lesion to develop into a substantial tumor mass [94]. Interestingly, iNKT activation via systemic injection of αGalCer significantly inhibited angiogenesis of intradermal tumors in mice, which depended on IFN-γ release by both iNKT cells and subsequently activated NK cells [95]. Even more interestingly, Dhodapkar et al showed that the neo-vasculature of glioma tumors expressed CD1d, identifying them as targets for this anti-angiogenic response [36]. It would therefore be interesting to determine neo-vasculature CD1d expression in more human tumors, to correlate this expression to activation status of infiltrated iNKT cells and other TILs and to the possible anti-angiogenic effect of iNKT cell activation (Figure 2B).

Finally, it has been proposed that once human colorectal cancers become clinically detectable, and thus have escaped early immune surveillance, the adaptive arm plays a predominant role in preventing disease progression. Pages et al reported that colorectal carcinoma tumors lacking the capacity for early invasion into the surrounding tissues (i.e. to form new vessels and to achieve lymphatic or perineural invasion) contained large numbers of Th1 oriented memory/ activated T cells in both the tumor center and in the tissue invasive margin [96]. In a follow-up study it was confirmed that patients with these tumors had a prolonged (disease specific) survival compared to patients with tumors that were not as well

infiltrated with these T cells [97]. In accordance with the observations described above that iNKT cells can infiltrate tumors, iNKT cells activated at the invasive margin of colorectal tumors could thus facilitate additional activation and proliferation of arriving T cells (Figure 2B). In addition iNKT could also locally inhibit angiogenesis in this setting.

Therapies aimed at the activation of iNKT cells in cancer patients

Recognizing the promise of iNKT cell activation in immunotherapy of cancer, attempts have been made to target them *in vivo* in clinical phase I studies, based on the use of αGalCer as a stimulatory ligand (summarized in Table 2). We established in 24 advanced cancer patients with solid tumors that 3 weekly intravenous injections of soluble αGalCer did not reach dose limiting toxicity over a wide dose range (50 – 4800 μg/ kg) [98]. Although no clinical responses were recorded, a transient increase in serum levels of immunostimulatory cytokines (IL-12, IFN-γ, TNF-α and GM-CSF) was observed after the first αGalCer injection in patients with relatively high iNKT cell levels. This was preceded by a rapid loss of detectable iNKT cells from the circulation within 24 h after αGalCer administration and iNKT cell levels remained low for up to 21 days after the first administration. In line with the decline in iNKT cell levels, no increase in serum cytokines was observed after a second and third injection with αGalCer (7 and 14 days after the first injection respectively). This might be explained by the relatively high dose of the glycolipid injected and the short interval between repeated injections. In a clinical phase I/II trial in chronic Hepatitis C patients we observed a re-appearance of circulating iNKT cells within 2 weeks after i.v. αGalCer given at a lower dose (0.1 – 10 μg/ kg). Furthermore, we observed a cytokine response even to a second and third αGalCer injection given at 4 and 8 weeks after the first injection respectively [99]. The lack of detectable iNKT cells quickly after i.v. injection of the glycolipid might reflect their antigen-specific activation by αGalCer, leading to TCR internalization, especially in the case of high dose αGalCer. This has been demonstrated previously in mice [100;101] and additional studies revealed that *in vivo* treatment with high dose soluble αGalCer can lead to long-term iNKT cell anergy, even after a single injection of the glycolipid [102;103].
The relatively low *in vivo* responsiveness of circulating iNKT cells upon i.v. injection of free αGalCer parallels the reduced *in vitro* responsiveness of iNKT cells when stimulated with autologous αGalCer loaded PBMC [32;34;42]. Both phenomena may be explained by the impaired function of APC in cancer patients. Analogous to the *in vitro* data which showed restored iNKT proliferation upon stimulation with αGalCer loaded, *in vitro* generated, fully functional mature DC [22], the injection of αGalCer pulsed DC gave rise to more potent *in vivo* expansion of endogenous iNKT cells. Three independent groups have now shown that this approach was well tolerated in humans. In two consecutive studies, the group of Nicol treated 4 [104] and 12 [105] patients respectively with metastatic carcinomas with autologous αGalCer pulsed immature moDC and found that this was well tolerated and resulted in a mild increase in circulating iNKT cell numbers. In the latter study, transient but potent pro-inflammatory effects were observed in peripheral blood samples after DC treatment, consisting of elevated levels of IL-12 and IFN-γ, reduced levels of IL-4, activation of T and NK cells and an increase in NK cell number and cytotoxicity. Again these phenomena were preceded by an abrupt loss of detectable iNKT cells from the circulation. Peripheral blood iNKT cell numbers normalized within 7 days after DC injection, however no expansion of iNKT cells was observed. The immunological effects were reproduced upon a second injection of immature moDC pulsed with αGalCer. Mature DC (i.e. immature DC that have been triggered by TLR ligands and inflammatory cytokines) are far more efficient in activating T cells and inducing their proliferation than immature DC [106] and *in vivo* activation of iNKT cells with properly matured DC is expected to be favorable. Indeed, Chang et al were able to induce dramatic expansion of circulating iNKT cells in 5 out of 5 advanced cancer patients who received i.v. injections with high purity, properly matured, αGalCer pulsed autologous moDC (> 100 fold expansion at peak level in all cases) [107]. Strikingly, despite having

undetectable peripheral blood iNKT cell counts at the time of study enrollment, the iNKT cell level remained above baseline after DC treatment for more than 85 days in all patients and for up to 6 months in two patients with longer follow up. In addition, the authors indirectly observed that iNKT cell activation was pursued by myeloid DC activation (elevated serum levels of myeloid DC associated soluble factors IL-12p40, MIP-1β and IP-10) and in line with this they detected an expansion of $CD8^+$ memory T cells specific for viral antigens (CMV). These findings strongly implicated an adjuvant effect on existing adaptive immunity. Ishikawa et al reported activation and transient expansion of resident iNKT cells in 3 out of 12 advanced lung cancer patients by injecting αGalCer pulsed DC [108]. However, their DC preparations were not of high purity but were in fact autologous PBMCs cultured for 7 to 14 days in the presence of GM-CSF, IL-2 and αGalCer. These preparations contained high levels of T cells and, given the protocol, most likely also substantial numbers of iNKT cells. A more controlled way of increasing the number of systemic iNKT cells in cancer patients is by the direct injection of *ex vivo* expanded autologous iNKT cells. Motohashi et al, who prepared autologous PBMC cultures for adoptive transfer derived from 6 non-small cell lung cancer patients by a method similar to the one Ishikawa et al used for the generation of APC, resulting in cell populations that were enriched for iNKT cells [109]. This approach was again well tolerated and resulted in a transient increase in circulating iNKT cells and direct *ex vivo* IFN-γ production in response to αGalCer in an ELISPOT assay.
It can be concluded from these clinical phase I studies that injection of preparations containing αGalCer pulsed DC and/ or iNKT cells is feasible, since it can be performed safely in advanced cancer patients and results in distinct activation of iNKT and downstream effector cells.

Part II: Towards autologous adoptive transfer of highly purified and well defined pro-inflammatory iNKT cells

Ex vivo expansion of iNKT cells from healthy controls and carcinoma patients

Although the phase I clinical trials performed thus far led to promising results there is ample room for improvement. When DC are used as "*in vivo* iNKT cell activators", a high iNKT cell expansion can be achieved, but the ability to control the functional aspects of the expanded iNKT cells *in vivo* (e.g. cytokine profile, capacity to home towards tumor sites or tumoricidal potential) is limited. For instance, in the clinical phase I study presented by Chang et al, iNKT cells that were isolated after the administration of αGalCer pulsed mature moDC appeared to be dysfunctional. Although these cells proliferated *in vitro* in response to αGalCer pulsed moDC, IFN-γ secretion in an αGalCer ELISPOT assay was inadequate [107]. In addition, the use of highly purified and well defined iNKT cells for adoptive transfer enables to ascribe any immunological or clinical effects observed to the injected iNKT cells. We have developed a method to expand peripheral blood iNKT cells of healthy controls as well as advanced cancer patients *in vitro*, and to polarize them towards a type 1 cytokine profile by stimulation of isolated TCR-Vα24$^+$ T cells with mature αGalCer pulsed moDC in the presence of IL-15 [22]. Using this method an up to 76 fold expansion was observed after one week of culture. These type 1 polarized iNKT cultures secreted large amounts of the pro-inflammatory cytokines IFN-γ, TNF-α and GM-CSF. Although some patient derived iNKT cell cultures showed an initial delay in proliferation, as reported previously [32;34;42], this could be overcome by repeated stimulation with αGalCer pulsed moDC, resulting in functionally competent iNKT cells. This suggests that even in patients with a putative state of iNKT cell anergy *in vitro* expansion of autologous iNKT cells using mature DC may allow for subsequent adoptive transfer of these cells.
Another advantage of autologous adoptive transfer of *ex vivo* expanded iNKT cells would be that their *in vitro* expansion and activation does not depend on autologous, often deficient DC. Autologous DC are not required, since CD1d is monomorphic. This allows for the use of DC cell lines as more standardized *in vitro* APC leading to more standardized iNKT cells for adoptive transfer. This would also facilitate the repeated stimulation of cultured iNKT cells *in vitro*, without requiring additional blood sampling of the patient to generate sufficient amounts of DC. We have previously demonstrated that human iNKT cells can be expanded using αGalCer pulsed DC derived from the CD34+ human acute myeloid leukemia derived cell line MUTZ-3 (M3-DC) [110]. In successive experiments we stimulated iNKT cells with M3-DC over-expressing IL-12 (M312-DC). The obtained cells had an increased activation phenotype, were capable of producing high levels of IFN-γ and could enhance tumor associated antigen (TAA) specific CTL priming by moDC *in vitro* (Bontkes HJ, *unpublished results*). M312-DC thus provides us with an attractive "off the shelf" DC source for the large scale expansion of functional iNKT cells from cancer patients.

Generation of mouse long-term high purity oligoclonal iNKT cell cultures using a DC line

Although the strategy of treating carcinoma patients with autologous adoptive transfer of *ex vivo* expanded iNKT cells is very appealing, long-term *in vitro* culture would be necessary in order to obtain sufficient numbers to repopulate patients with iNKT cells upto healthy control levels. This would especially be the case in those individuals who would probably benefit most from this therapy, namely those with a severe deficiency in circulating iNKT cells and/ or a poor *in vitro* proliferative response towards αGalCer. However, the effect of long-term *in vitro* stimulation with DC pulsed with the strong agonist αGalCer on *in vivo* iNKT cell functionality has not previously been investigated.

We therefore developed a method to generate long-term high purity oligoclonal mouse iNKT cell cultures representative of *in vivo* iNKT cells. These were used in different experiments to determine whether their *in vitro* functionality and, more importantly, their capacity to enhance anti-tumor responses *in vivo* remained unaffected. Cells from the immature DC line D1 [111] were treated shortly with LPS and IFN-γ and pulsed with αGalCer to generate mature DC, capable of facilitating the *in vitro* expansion of iNKT cells isolated from mouse spleen [112]. This expansion was especially potent when IL-7 was added to the cultures during weekly re-stimulation with mature D1 DC pulsed with αGalCer. Although other studies demonstrated that it is possible to generate from mice either short lived cell cultures containing bona fide iNKT cells using spleen or bone marrow derived DC and αGalCer [51;113;114], or long lived clone derived iNKT cell hybridomas [115-117], our study was the first to make available large scale, highly pure long-term oligoclonal mouse iNKT cell lines representative of *in vivo* iNKT cells.
Using αGalCer pulsed mature D1 DC and IL-7, we typically obtained 10^8 iNKT cells from one mouse spleen within 10-14 weeks of culture and these cultures could be maintained for over 24 months. The iNKT cell cultures consisted of both $CD4^+$ and $CD4^-$ cells and resembled wild-type iNKT cells since they all could bind to αGalCer loaded mouse CD1d:IgG1 dimers and could readily release substantial amounts of IFN-γ and IL-4 upon stimulation with αGalCer pulsed CD1d transfected HeLa cells (HeLa-CD1d). Furthermore, after 24 hours of stimulation with αGalCer pulsed HeLa-CD1d all iNKT cell lines tested had secreted IFN-γ, GM-CSF, IL-4, IL-5, IL-6, IL-10 and IL-13 reflecting the dichotomous regulatory capacity of *in vivo* iNKT cells [5;15;16]. From these findings we concluded that even after long-term chronic antigenic stimulation *in vitro* mouse iNKT cells retain their most appreciated functional aspect, namely they can recognize αGalCer/ CD1d complexes and respond accordingly by releasing substantial amounts of Th1 and Th2 cytokines.

Retained capacity of long term mouse iNKT cell cultures to enhance in vivo immune response against experimental tumor metastases

As mentioned earlier in this review, mouse iNKT cells can enhance anti-tumor responses depending on the innate [23-27] as well as the adaptive [30] arm of the immune system. We investigated the capacity of iNKT cells obtained by *in vitro* culture with αGalCer pulsed D1 DC to enhance protection against B16.F10 lung metastases, upon their adoptive transfer into Wild-type mice shortly after tumor injection (Molling JW et al, *manuscript submitted*). Intravenous injection of 4 out of 4 lines investigated evoked a systemic cytokine storm (elevated IFN-γ and IL-4 in splenocytes irrespective of *in vitro* re-stimulation) that lasted for up to 11 days. Furthermore iNKT cell transfer resulted in a preferential influx of NK cells into the lungs of these mice and, most importantly, an augmented NK cell mediated protection against B16.F10 experimental lung metastases.
Crowe et al investigated the effect of transfer of freshly isolated iNKT cells on B16.F10 lung metastases in iNKT cell deficient mice. In their study, the activation of NK cells and thus the inhibition of metastasis formation required additional treatment of mice with αGalCer. We demonstrated that additional αGalCer injection was not required when iNKT cells had been activated *in vitro* with αGalCer pulsed D1 DC [118]. This is in line with the findings by Shin et al, who observed inhibition of experimental B16.F10 liver metastases upon adoptive transfer of *in vitro* IL-12 pre-activated iNKT cells into iNKT deficient mice without additional αGalCer treatment [119]. Interestingly, Shin et al also demonstrated that iNKT cell transfer mediated protection was superior to injection of high dose IFN-γ or a combination of high dose IFN-γ, IL-2 and IL-4. This protection is possibly due to a selective recruitment of iNKT cells to the tumor site followed by their local release of chemokines and cytokines, allowing for the recruitment and trans-activation of downstream effector cells. Although direct evidence for this is still lacking, we did observe that long-term iNKT cell lines can express the “tumor homing receptors” CXCR3 and CCR5 (Molling JW, *unpublished results*) and that they

secrete a broad variety of cytokines *in vitro* (as described above [112]). These observations, in combination with the finding that NK cells were selectively recruited to the lungs after i.v. iNKT injection, are in favor of the sequential influx of iNKT cells and NK cells into the lungs of B16.F10 tumor challenged mice.
In summary, we demonstrated that adoptive transfer of long-term high purity oligoclonal mouse iNKT cells, that were proven to maintain their *in vitro* functionality, lead to enhanced immune protection against an experimental tumor that was capable of evading immunity in Wild-type mice. In the B16.F10 lung metastasis model we used, this was achieved via the trans-activation of NK cells. This function of the iNKT cells was retained even after more than 24 months of weekly stimulation using D1 DC pulsed with αGalCer. The potential of autologous adoptive transfer of *ex vivo* expanded iNKT cells as an immunotherapeutic strategy for the treatment of cancer patients is illustrated by these pre-clinical studies.

Concluding remarks

Based on the epidemiologic and preclinical data reviewed here, re-constitution of the circulating iNKT cell pool as an immunotherapeutic adjuvant therapy of cancer appears to be feasible. In addition, screening for those patients who are severely deficient in circulating iNKT cells would identify them as the individuals who could benefit the most from this approach. In our view, the autologous adoptive transfer of *ex vivo* expanded iNKT cells provides a promising strategy. An advantage above adoptive transfer of e.g. TAA pulsed DC vaccines [120], or transfer of TAA specific T cells generated *in vitro* via expansion using TAA pulsed DC or via TCR gene transfer [121;122] is their universal immunostimulating potential. Therefore no pre-selection for patients carrying a certain HLA type or expressing a particular tumor antigen is necessary. The risk of selection of TAA and/ or HLA negative tumor cells is expected to be low, compared to TAA peptide based immunotherapy. Furthermore, safety issues always need to be addressed with the application of TCR gene transfer because of the use of retroviral vectors [122].
iNKT cells can be expanded *in vitro* using the “off the shelve” Mutz-3 cell line derived DC pulsed with αGalCer. Using this *ex vivo* approach, high purity iNKT cells can be obtained and their potential to enhance anti tumor responses can be strengthened by defining the desired culture conditions (e.g. the use of IL-12 over expressing Mutz-3 DC). More importantly, their capacity to secrete high amounts of (type 1) cytokines (e.g. IFN-γ and GM-CSF, to enhance the functionality of other immune cells (e.g. DC, antigen specific CTL or NK cells), or their expression of “tumor homing receptors” (e.g. CXCR3 and CCR5 for carcinomas or CCR2 for neuroblastomas) can be checked prior to adoptive transfer.
There are many questions remaining that could lead to a more efficient therapeutic outcome when answered. Some examples are the following: How do peripheral and intra-tumor iNKT cell numbers and function relate? What is the impact of systemic or local (tumor site) enhancement of iNKT cell numbers on (tumor infiltrating) DC, T and NK cell function, also with regard to disease outcome? Does additional treatment with αGalCer enhance these responses and should it then be administered systemically (e.g. loaded on DC) or locally? Can the responses be enhanced when the negative influence on anti-tumor responses of nTreg is abrogated (e.g. by blocking CTLA4 function [123-126] or by toll-like-receptor 2 triggering [127;128])?
Nonetheless, we already observed that the amount of pre-therapy circulating iNKT cells has a dramatic impact on disease specific survival upon curative radiotherapy of HNSCC patients. Immuno-adjuvant treatment of iNKT cell deficient HNSCC patients, by radiotherapy accompanied by reconstitution of their peripheral blood iNKT cell compartment using autologous adoptive transfer of iNKT cells, could provide a solid base from which questions as those above could be further addressed.

Figure legends

Figure 1: *Proposed mechanism of CD1d up-regulation in the tumor micro-environment as target for iNKT cells*
A) Cartoon of steady state epithelial tissue and an adjacent (lymphatic) venule consisting of epithelial (EPI) and endothelial (ENDO) cells respectively, expressing low levels of CD1d. Antigen presenting cells (APC) reside within the epithelial layer and have only marginal to absent CD1d expression. B) Early event in carcinogenesis: pre-malignant transformation of epithelial cells (PRE-MALIGN) does not alter CD1d expression. C) Late event in carcinogenesis: strongly and invasively growing carcinoma cells (TUMOR) release high levels of heat shock proteins (Hsp) and induce damage in the surrounding tissue. Damaged epithelial cells (DAMAGE) release additional Hsp. D) Strong up-regulation of CD1d on tumor cells, damaged epithelial cells and APC within the tumor micro-environment in response to elevated Hsp levels provide multiple candidate targets for auto-reactive invariant natural killer T cells.

Figure 2: *Proposed role of iNKT cells in enhancing immune function in tumor microenvironment*
A) Chemokine (CHEM) release from the tumor micro-environment results in the influx of invariant natural killer T (iNKT) which subsequently co-localize with residing immature dendritic cells (immDC) that might be kept in an immature state by tumor derived soluble factors. Binding of the iNKT TCR to the APC CD1d, presenting endogenous glycolipids triggers IFN-γ release by the iNKT and IL-12 release by the immDC, creating a positive feedback loop that results in elevated iNKT derived IFN-γ and DC maturation. As a result, mature DC (matDC) up-regulate CCR7 and migrate towards the tumor draining lymph node, where they can prime $CD4^+$ T helper cells and $CD8^+$ cytotoxic T lymphocytes and facilitate B cell activation. B) iNKT cells recognize endogenous glycolipids bound to CD1d on tumor cells, damaged epithelial cells or APC and subsequently release IFN-γ locally. As a result chemokine release is enhanced, facilitating considerable influx of anti-tumor effector cells. Natural killer (NK) cells and $CD8^+$ cytotoxic T lymphocytes (CTL) arrive at the tumor site and can i) cross the endothelial barrier to directly kill the tumor cells. ii) iNKT cells that are activated by CD1d on endothelial cells secrete IFN-γ to activate NK cells that subsequently release higher levels of IFN-γ, resulting in the inhibition of neo-angiogenesis initiated by the developing tumor. iii) iNKT cells in the (lymphatic) circulation recognize, and are activated by, CD1d on migrating tumor cells. IFN-γ, released by the activated iNKT cells enhances antibody dependent cellular cytotoxicity (ADCC) mediated by NK cells and antibodies specific for cell surface tumor associated antigens (Ig/ Fc-R).

References

(1) Penn I. Tumors of the immunocompromised patient. Annu Rev Med 1988; 39:63-73.

(2) Whiteside TL. Immune suppression in cancer: effects on immune cells, mechanisms and future therapeutic intervention. Semin Cancer Biol 2006; 16(1):3-15.

(3) Young MR. Protective mechanisms of head and neck squamous cell carcinomas from immune assault. Head Neck 2006; 28(5):462-470.

(4) Whiteside TL. The role of immune cells in the tumor microenvironment. Cancer Treat Res 2006; 130:103-124.

(5) Godfrey DI, Hammond KJ, Poulton LD, Smyth MJ, Baxter AG. NKT cells: facts, functions and fallacies. Immunol Today 2000; 21(11):573-583.

(6) Porcelli S, Yockey CE, Brenner MB, Balk SP. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8- alpha/beta T cells demonstrates preferential use of several V beta genes and an invariant TCR alpha chain. J Exp Med 1993; 178(1):1-16.

(7) Spada FM, Koezuka Y, Porcelli SA. CD1d-restricted recognition of synthetic glycolipid antigens by human natural killer T cells. J Exp Med 1998; 188(8):1529-1534.

(8) Speak AO, Salio M, Neville DC, Fontaine J, Priestman DA, Platt N et al. Implications for invariant natural killer T cell ligands due to the restricted presence of isoglobotrihexosylceramide in mammals. Proc Natl Acad Sci U S A 2007; 104(14):5971-5976.

(9) Porubsky S, Speak AO, Luckow B, Cerundolo V, Platt FM, Grone HJ. Normal development and function of invariant natural killer T cells in mice with isoglobotrihexosylceramide (iGb3) deficiency. Proc Natl Acad Sci U S A 2007; 104(14):5977-5982.

(10) Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. Science 1997; 278(5343):1626-1629.

(11) Wu DY, Segal NH, Sidobre S, Kronenberg M, Chapman PB. Cross-presentation of disialoganglioside GD3 to natural killer T cells. J Exp Med 2003; 198(1):173-181.

(12) Zhou D, Mattner J, Cantu C, III, Schrantz N, Yin N, Gao Y et al. Lysosomal glycosphingolipid recognition by NKT cells. Science 2004; 306(5702):1786-1789.

(13) Kinjo Y, Wu D, Kim G, Xing GW, Poles MA, Ho DD et al. Recognition of bacterial glycosphingolipids by natural killer T cells. Nature 2005; 434(7032):520-525.

(14) Mattner J, Debord KL, Ismail N, Goff RD, Cantu C, III, Zhou D et al. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. Nature 2005; 434(7032):525-529.

(15) van der Vliet HJ, Molling JW, von Blomberg BM, Nishi N, Kolgen W, van den Eertwegh AJ et al. The immunoregulatory role of CD1d-restricted natural killer T cells in disease. Clin Immunol 2004; 112(1):8-23.

(16) Gumperz JE, Miyake S, Yamamura T, Brenner MB. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. J Exp Med 2002; 195(5):625-636.

(17) Kim CH, Butcher EC, Johnston B. Distinct subsets of human Valpha24-invariant NKT cells: cytokine responses and chemokine receptor expression. Trends Immunol 2002; 23(11):516-519.

(18) Lee PT, Benlagha K, Teyton L, Bendelac A. Distinct functional lineages of human V(alpha)24 natural killer T cells. J Exp Med 2002; 195(5):637-641.

(19) Seino K, Taniguchi M. Functionally distinct NKT cell subsets and subtypes. J Exp Med 2005; 202(12):1623-1626.

(20) Lin H, Nieda M, Rozenkov V, Nicol AJ. Analysis of the effect of different NKT cell subpopulations on the activation of CD4 and CD8 T cells, NK cells, and B cells. Exp Hematol 2006; 34(3):289-295.

(21) Osada T, Morse MA, Lyerly HK, Clay TM. Ex vivo expanded human CD4+ regulatory NKT cells suppress expansion of tumor antigen-specific CTLs. Int Immunol 2005; 17(9):1143-1155.

(22) van der Vliet HJ, Molling JW, Nishi N, Masterson AJ, Kolgen W, Porcelli SA et al. Polarization of Valpha24+ Vbeta11+ natural killer T cells of healthy volunteers and cancer patients using alpha-galactosylceramide-loaded and environmentally instructed dendritic cells. Cancer Res 2003; 63(14):4101-4106.

(23) Crowe NY, Smyth MJ, Godfrey DI. A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. J Exp Med 2002; 196(1):119-127.

(24) Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Sato H et al. Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Valpha14 NKT cells. Proc Natl Acad Sci U S A 1998; 95(10):5690-5693.

(25) Nakagawa R, Nagafune I, Tazunoki Y, Ehara H, Tomura H, Iijima R et al. Mechanisms of the antimetastatic effect in the liver and of the hepatocyte injury induced by alpha-galactosylceramide in mice. J Immunol 2001; 166(11):6578-6584.

(26) Nakui M, Ohta A, Sekimoto M, Sato M, Iwakabe K, Yahata T et al. Potentiation of antitumor effect of NKT cell ligand, alpha-galactosylceramide by combination with IL-12 on lung metastasis of malignant melanoma cells. Clin Exp Metastasis 2000; 18(2):147-153.

(27) Smyth MJ, Wallace ME, Nutt SL, Yagita H, Godfrey DI, Hayakawa Y. Sequential activation of NKT cells and NK cells provides effective innate immunotherapy of cancer. J Exp Med 2005; 201(12):1973-1985.

(28) Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. J Exp Med 2003; 198(2):267-279.

(29) Hermans IF, Silk JD, Gileadi U, Salio M, Mathew B, Ritter G et al. NKT cells enhance CD4+ and CD8+ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells. J Immunol 2003; 171(10):5140-5147.

(30) Liu K, Idoyaga J, Charalambous A, Fujii S, Bonito A, Mordoh J et al. Innate NKT lymphocytes confer superior adaptive immunity via tumor-capturing dendritic cells. J Exp Med 2005; 202(11):1507-1516.

(31) Kawano T, Nakayama T, Kamada N, Kaneko Y, Harada M, Ogura N et al. Antitumor cytotoxicity mediated by ligand-activated human V alpha24 NKT cells. Cancer Res 1999; 59(20):5102-5105.

(32) Tahir SM, Cheng O, Shaulov A, Koezuka Y, Bubley GJ, Wilson SB et al. Loss of IFN-gamma production by invariant NK T cells in advanced cancer. J Immunol 2001; 167(7):4046-4050.

(33) Motohashi S, Kobayashi S, Ito T, Magara KK, Mikuni O, Kamada N et al. Preserved IFN-alpha production of circulating Valpha24 NKT cells in primary lung cancer patients. Int J Cancer 2002; 102(2):159-165.

(34) Konishi J, Yamazaki K, Yokouchi H, Shinagawa N, Iwabuchi K, Nishimura M. The characteristics of human NKT cells in lung cancer--CD1d independent cytotoxicity against lung cancer cells by NKT cells and decreased human NKT cell response in lung cancer patients. Hum Immunol 2004; 65(11):1377-1388.

(35) Dhodapkar MV, Geller MD, Chang DH, Shimizu K, Fujii S, Dhodapkar KM et al. A reversible defect in natural killer T cell function characterizes the progression of premalignant to malignant multiple myeloma. J Exp Med 2003; 197(12):1667-1676.

(36) Dhodapkar KM, Cirignano B, Chamian F, Zagzag D, Miller DC, Finlay JL et al. Invariant natural killer T cells are preserved in patients with glioma and exhibit antitumor lytic activity following dendritic cell-mediated expansion. Int J Cancer 2004; 109(6):893-899.

(37) Metelitsa LS, Wu HW, Wang H, Yang Y, Warsi Z, Asgharzadeh S et al. Natural Killer T Cells Infiltrate Neuroblastomas Expressing the Chemokine CCL2. J Exp Med 2004; 199(9):1213-1221.

(38) Delarosa O, Tarazona R, Casado JG, Alonso C, Ostos B, Pena J et al. Valpha24+ NKT cells are decreased in elderly humans. Exp Gerontol 2002; 37(2-3):213-217.

(39) Sandberg JK, Bhardwaj N, Nixon DF. Dominant effector memory characteristics, capacity for dynamic adaptive expansion, and sex bias in the innate Valpha24 NKT cell compartment. Eur J Immunol 2003; 33(3):588-596.

(40) Molling JW, Kolgen W, van der Vliet HJ, Boomsma MF, Kruizenga H, Smorenburg CH et al. Peripheral blood IFN-gamma-secreting Valpha24+Vbeta11+ NKT cell numbers are decreased in cancer patients independent of tumor type or tumor load. Int J Cancer 2005; 116(1):87-93.

(41) Crough T, Purdie DM, Okai M, Maksoud A, Nieda M, Nicol AJ. Modulation of human Valpha24(+)Vbeta11(+) NKT cells by age, malignancy and conventional anticancer therapies. Br J Cancer 2004; 91(11):1880-1886.

(42) Yanagisawa K, Seino K, Ishikawa Y, Nozue M, Todoroki T, Fukao K. Impaired proliferative response of V alpha 24 NKT cells from cancer patients against alpha-galactosylceramide. J Immunol 2002; 168(12):6494-6499.

(43) Peralbo E, Delarosa O, Gayoso I, Pita ML, Tarazona R, Solana R. Decreased frequency and proliferative response of invariant Valpha24Vbeta11 natural killer T (iNKT) cells in healthy elderly. Biogerontology 2006; 7(5-6):483-492.

(44) Hoffmann TK, Muller-Berghaus J, Ferris RL, Johnson JT, Storkus WJ, Whiteside TL. Alterations in the frequency of dendritic cell subsets in the peripheral circulation of patients with squamous cell carcinomas of the head and neck. Clin Cancer Res 2002; 8(6):1787-1793.

(45) Azuma T, Takahashi T, Kunisato A, Kitamura T, Hirai H. Human CD4+ CD25+ regulatory T cells suppress NKT cell functions. Cancer Res 2003; 63(15):4516-4520.

(46) Wolf AM, Wolf D, Steurer M, Gastl G, Gunsilius E, Grubeck-Loebenstein B. Increase of regulatory T cells in the peripheral blood of cancer patients. Clin Cancer Res 2003; 9(2):606-612.

(47) Okita R, Saeki T, Takashima S, Yamaguchi Y, Toge T. CD4+CD25+ regulatory T cells in the peripheral blood of patients with breast cancer and non-small cell lung cancer. Oncol Rep 2005; 14(5):1269-1273.

(48) Ichihara F, Kono K, Takahashi A, Kawaida H, Sugai H, Fujii H. Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. Clin Cancer Res 2003; 9(12):4404-4408.

(49) Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G et al. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. J Immunol 2002; 169(5):2756-2761.

(50) Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol 2002; 2(6):389-400.

(51) Chamoto K, Takeshima T, Kosaka A, Tsuji T, Matsuzaki J, Togashi Y et al. NKT cells act as regulatory cells rather than killer cells during activation of NK cell-mediated cytotoxicity by alpha-galactosylceramide in vivo. Immunol Lett 2004; 95(1):5-11.

(52) van der Vliet HJ, Pinedo HM, von Blomberg BM, van den Eertwegh AJ, Scheper RJ, Giaccone G. Natural Killer T cells. Lancet Oncol 2002; 3(9):574.

(53) van der Vliet HJ, Nishi N, Koezuka Y, von Blomberg BM, van den Eertwegh AJ, Porcelli SA et al. Potent expansion of human natural killer T cells using alpha-galactosylceramide (KRN7000)-loaded monocyte-derived dendritic cells, cultured in the presence of IL-7 and IL-15. J Immunol Methods 2001; 247(1-2):61-72.

(54) Nishi N, van der Vliet HJ, Koezuka Y, von Blomberg BM, Scheper RJ, Pinedo HM et al. Synergistic effect of KRN7000 with interleukin-15, -7, and -2 on the expansion of human V alpha 24+V beta 11+ T cells in vitro. Hum Immunol 2000; 61(4):357-365.

(55) Metelitsa LS, Naidenko OV, Kant A, Wu HW, Loza MJ, Perussia B et al. Human NKT cells mediate antitumor cytotoxicity directly by recognizing target cell CD1d with bound ligand or indirectly by producing IL-2 to activate NK cells. J Immunol 2001; 167(6):3114-3122.

(56) Takahashi T, Nieda M, Koezuka Y, Nicol A, Porcelli SA, Ishikawa Y et al. Analysis of human V alpha 24+ CD4+ NKT cells activated by alpha-glycosylceramide-pulsed monocyte-derived dendritic cells. J Immunol 2000; 164(9):4458-4464.

(57) Molling JW, Langius JA, Langendijk JA, Leemans CR, Bontkes HJ, van der Vliet HJ et al. Low levels of circulating invariant natural killer T cells predict poor clinical outcome in patients with head and neck squamous cell carcinoma. J Clin Oncol 2007; 25(7):862-868.

(58) Soysal V, Yigitbasi OG, Alper M, Patiroglu T, Guney E. Total lymphocyte and T lymphocyte subpopulation levels in head and neck squamous cell carcinomas. J Exp Clin Cancer Res 1998; 17(2):207-212.

(59) Wolf GT, Bradford CR, Urba S, Smith A, Eisbruch A, Chepeha DB et al. Immune reactivity does not predict chemotherapy response, organ preservation, or survival in advanced laryngeal cancer. Laryngoscope 2002; 112(8 Pt 1):1351-1356.

(60) Kuss I, Hathaway B, Ferris RL, Gooding W, Whiteside TL. Decreased absolute counts of T lymphocyte subsets and their relation to disease in squamous cell carcinoma of the head and neck. Clin Cancer Res 2004; 10(11):3755-3762.

(61) Schantz SP, Shillitoe EJ, Brown B, Campbell B. Natural killer cell activity and head and neck cancer: a clinical assessment. J Natl Cancer Inst 1986; 77(4):869-875.

(62) Schantz SP, Ordonez NG. Quantitation of natural killer cell function and risk of metastatic poorly differentiated head and neck cancer. Nat Immun Cell Growth Regul 1991; 10(5):278-288.

(63) Gonzalez FM, Vargas JA, Lopez-Cortijo C, Castejon R, Gorriz C, Ramirez-Camacho R et al. Prognostic significance of natural killer cell activity in patients with laryngeal carcinoma. Arch Otolaryngol Head Neck Surg 1998; 124(8):852-856.

(64) Reichert TE, Day R, Wagner EM, Whiteside TL. Absent or low expression of the zeta chain in T cells at the tumor site correlates with poor survival in patients with oral carcinoma. Cancer Res 1998; 58(23):5344-5347.

(65) Heimdal JH, Aarstad HJ, Olofsson J. Peripheral blood T-lymphocyte and monocyte function and survival in patients with head and neck carcinoma. Laryngoscope 2000; 110(3 Pt 1):402-407.

(66) Der Vliet HJ, Nishi N, De Gruijl TD, von Blomberg BM, van den Eertwegh AJ, Pinedo HM et al. Human natural killer T cells acquire a memory-activated phenotype before birth. Blood 2000; 95(7):2440-2442.

(67) Park SH, Benlagha K, Lee D, Balish E, Bendelac A. Unaltered phenotype, tissue distribution and function of Valpha14(+) NKT cells in germ-free mice. Eur J Immunol 2000; 30(2):620-625.

(68) Thomas SY, Hou R, Boyson JE, Means TK, Hess C, Olson DP et al. CD1d-restricted NKT cells express a chemokine receptor profile indicative of Th1-type inflammatory homing cells. J Immunol 2003; 171(5):2571-2580.

(69) Kim CH, Johnston B, Butcher EC. Trafficking machinery of NKT cells: shared and differential chemokine receptor expression among V alpha 24(+)V beta 11(+) NKT cell subsets with distinct cytokine-producing capacity. Blood 2002; 100(1):11-16.

(70) Apostolou I, Takahama Y, Belmant C, Kawano T, Huerre M, Marchal G et al. Murine natural killer T(NKT) cells contribute to the granulomatous reaction caused by mycobacterial cell walls. Proc Natl Acad Sci U S A 1999; 96(9):5141-5146.

(71) Kawakami K, Kinjo Y, Uezu K, Yara S, Miyagi K, Koguchi Y et al. Monocyte chemoattractant protein-1-dependent increase of V alpha 14 NKT cells in lungs and their roles in Th1 response and host defense in cryptococcal infection. J Immunol 2001; 167(11):6525-6532.

(72) Seeger RC, Brodeur GM, Sather H, Dalton A, Siegel SE, Wong KY et al. Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. N Engl J Med 1985; 313(18):1111-1116.

(73) Song L, Ara T, Wu HW, Woo CW, Reynolds CP, Seeger RC et al. Oncogene MYCN regulates localization of NKT cells to the site of disease in neuroblastoma. J Clin Invest 2007; 117(9):2702-2712.

(74) Tachibana T, Onodera H, Tsuruyama T, Mori A, Nagayama S, Hiai H et al. Increased intratumor Valpha24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas. Clin Cancer Res 2005; 11(20):7322-7327.

(75) Ohtani H. Focus on TILs: prognostic significance of tumor infiltrating lymphocytes in human colorectal cancer. Cancer Immun 2007; 7:4.

(76) Musha H, Ohtani H, Mizoi T, Kinouchi M, Nakayama T, Shiiba K et al. Selective infiltration of CCR5(+)CXCR3(+) T lymphocytes in human colorectal carcinoma. Int J Cancer 2005; 116(6):949-956.

(77) Colgan SP, Pitman RS, Nagaishi T, Mizoguchi A, Mizoguchi E, Mayer LF et al. Intestinal heat shock protein 110 regulates expression of CD1d on intestinal epithelial cells. J Clin Invest 2003; 112(5):745-754.

(78) Nicchitta CV. Come forth CD1d: Hsp110 in the regulation of intestinal epithelial CD1d expression. J Clin Invest 2003; 112(5):646-648.

(79) Bobryshev YV, Lord RS. Expression of heat shock protein-70 by dendritic cells in the arterial intima and its potential significance in atherogenesis. J Vasc Surg 2002; 35(2):368-375.

(80) Bonish B, Jullien D, Dutronc Y, Huang BB, Modlin R, Spada FM et al. Overexpression of CD1d by keratinocytes in psoriasis and CD1d-dependent IFN-gamma production by NK-T cells. J Immunol 2000; 165(7):4076-4085.

(81) Curry JL, Qin JZ, Bonish B, Carrick R, Bacon P, Panella J et al. Innate immune-related receptors in normal and psoriatic skin. Arch Pathol Lab Med 2003; 127(2):178-186.

(82) Isomoto H, Oka M, Yano Y, Kanazawa Y, Soda H, Terada R et al. Expression of heat shock protein (Hsp) 70 and Hsp 40 in gastric cancer. Cancer Lett 2003; 198(2):219-228.

(83) Sasaki J, Dejehansart M, De Bruyn J. The expression of mycobacterial heat shock protein (HSP64) on Meth A tumour cells. Immunol Cell Biol 1994; 72(5):415-418.

(84) Feng H, Zeng Y, Whitesell L, Katsanis E. Stressed apoptotic tumor cells express heat shock proteins and elicit tumor-specific immunity. Blood 2001; 97(11):3505-3512.

(85) Kim KK, Jang TJ, Kim JR. HSP70 and ER expression in cervical intraepithelial neoplasia and cervical cancer. J Korean Med Sci 1998; 13(4):383-388.

(86) Yue SC, Shaulov A, Wang R, Balk SP, Exley MA. CD1d ligation on human monocytes directly signals rapid NF-{kappa}B activation and production of bioactive IL-12. Proc Natl Acad Sci U S A 2005; 102(33):11811-11816.

(87) Eberl G, Brawand P, MacDonald HR. Selective bystander proliferation of memory CD4+ and CD8+ T cells upon NK T or T cell activation. J Immunol 2000; 165(8):4305-4311.

(88) Gorbachev AV, Fairchild RL. Activated NKT cells increase dendritic cell migration and enhance CD8+ T cell responses in the skin. Eur J Immunol 2006; 36(9):2494-2503.

(89) Montoya CJ, Jie HB, Al Harthi L, Mulder C, Patino PJ, Rugeles MT et al. Activation of plasmacytoid dendritic cells with TLR9 agonists initiates invariant NKT cell-mediated cross-talk with myeloid dendritic cells. J Immunol 2006; 177(2):1028-1039.

(90) Vincent MS, Leslie DS, Gumperz JE, Xiong X, Grant EP, Brenner MB. CD1-dependent dendritic cell instruction. Nat Immunol 2002; 3(12):1163-1168.

(91) Vuylsteke RJ, Molenkamp BG, van Leeuwen PA, Meijer S, Wijnands PG, Haanen JB et al. Tumor-specific CD8+ T cell reactivity in the sentinel lymph node of GM-CSF-treated stage I melanoma patients is associated with high myeloid dendritic cell content. Clin Cancer Res 2006; 12(9):2826-2833.

(92) Galli G, Pittoni P, Tonti E, Malzone C, Uematsu Y, Tortoli M et al. Invariant NKT cells sustain specific B cell responses and memory. Proc Natl Acad Sci U S A 2007; 104(10):3984-3989.

(93) Lin H, Nieda M, Rozenkov V, Nicol AJ. Analysis of the effect of different NKT cell subpopulations on the activation of CD4 and CD8 T cells, NK cells, and B cells. Exp Hematol 2006; 34(3):289-295.

(94) Nishida N, Yano H, Nishida T, Kamura T, Kojiro M. Angiogenesis in cancer. Vasc Health Risk Manag 2006; 2(3):213-219.

(95) Hayakawa Y, Takeda K, Yagita H, Smyth MJ, Van Kaer L, Okumura K et al. IFN-gamma-mediated inhibition of tumor angiogenesis by natural killer T- cell ligand, alpha-galactosylceramide. Blood 2002; 100(5):1728-1733.

(96) Pages F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R et al. Effector memory T cells, early metastasis, and survival in colorectal cancer. N Engl J Med 2005; 353(25):2654-2666.

(97) Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006; 313(5795):1960-1964.

(98) Giaccone G, Punt CJ, Ando Y, Ruijter R, Nishi N, Peters M et al. A Phase I Study of the Natural Killer T-Cell Ligand alpha- Galactosylceramide (KRN7000) in Patients with Solid Tumors. Clin Cancer Res 2002; 8(12):3702-3709.

(99) Veldt BJ, van der Vliet HJ, von Blomberg BM, van Vlierberghe H, Gerken G, Nishi N et al. Randomized placebo controlled phase I/II trial of alpha-galactosylceramide for the treatment of chronic hepatitis C. J Hepatol 2007; 47(3):356-365.

(100) Crowe NY, Uldrich AP, Kyparissoudis K, Hammond KJ, Hayakawa Y, Sidobre S et al. Glycolipid Antigen Drives Rapid Expansion and Sustained Cytokine Production by NK T Cells. J Immunol 2003; 171(8):4020-4027.

(101) Wilson MT, Johansson C, Olivares-Villagomez D, Singh AK, Stanic AK, Wang CR et al. The response of natural killer T cells to glycolipid antigens is characterized by surface receptor down-modulation and expansion. Proc Natl Acad Sci U S A 2003; 100(19):10913-10918.

(102) Parekh VV, Wilson MT, Olivares-Villagomez D, Singh AK, Wu L, Wang CR et al. Glycolipid antigen induces long-term natural killer T cell anergy in mice. J Clin Invest 2005; 115(9):2572-2583.

(103) Uldrich AP, Crowe NY, Kyparissoudis K, Pellicci DG, Zhan Y, Lew AM et al. NKT cell stimulation with glycolipid antigen in vivo: costimulation-dependent expansion, Bim-dependent contraction, and hyporesponsiveness to further antigenic challenge. J Immunol 2005; 175(5):3092-3101.

(104) Okai M, Nieda M, Tazbirkova A, Horley D, Kikuchi A, Durrant S et al. Human peripheral blood Valpha24+ Vbeta11+ NKT cells expand following administration of alpha-galactosylceramide-pulsed dendritic cells. Vox Sang 2002; 83(3):250-253.

(105) Nieda M, Okai M, Tazbirkova A, Lin H, Yamaura A, Ide K et al. Therapeutic activation of V{alpha}24+V{beta}11+NKT cells in human subjects results in highly co-ordinated secondary activation of acquired and innate immunity. Blood 2003.

(106) Cella M, Sallusto F, Lanzavecchia A. Origin, maturation and antigen presenting function of dendritic cells. Curr Opin Immunol 1997; 9(1):10-16.

(107) Chang DH, Osman K, Connolly J, Kukreja A, Krasovsky J, Pack M et al. Sustained expansion of NKT cells and antigen-specific T cells after injection of alpha-galactosyl-ceramide loaded mature dendritic cells in cancer patients. J Exp Med 2005; 201(9):1503-1517.

(108) Ishikawa A, Motohashi S, Ishikawa E, Fuchida H, Higashino K, Otsuji M et al. A phase I study of alpha-galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent non-small cell lung cancer. Clin Cancer Res 2005; 11(5):1910-1917.

(109) Motohashi S, Ishikawa A, Ishikawa E, Otsuji M, Iizasa T, Hanaoka H et al. A Phase I Study of In vitro Expanded Natural Killer T Cells in Patients with Advanced and Recurrent Non-Small Cell Lung Cancer. Clin Cancer Res 2006.

(110) Masterson AJ, Sombroek CC, de Gruijl TD, Graus YM, van der Vliet HJ, Lougheed SM et al. MUTZ-3, a human cell line model for the cytokine-induced differentiation of dendritic cells from CD34+ precursors. Blood 2002; 100(2):701-703.

(111) Winzler C, Rovere P, Rescigno M, Granucci F, Penna G, Adorini L et al. Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. J Exp Med 1997; 185(2):317-328.

(112) Molling JW, Moreno M, van der Vliet HJ, von Blomberg BM, van den Eertwegh AJ, Scheper RJ et al. Generation and sustained expansion of mouse spleen invariant NKT cell lines with preserved cytokine releasing capacity. J Immunol Methods 2007; 322(1-2):70-81.

(113) Maeda M, Lohwasser S, Yamamura T, Takei F. Regulation of NKT cells by Ly49: analysis of primary NKT cells and generation of NKT cell line. J Immunol 2001; 167(8):4180-4186.

(114) Ikarashi Y, Iizuka A, Heike Y, Yoshida M, Takaue Y, Wakasugi H. Cytokine production and migration of in vitro-expanded NK1.1(-) invariant Valpha14 natural killer T (Valpha14i NKT) cells using alpha-galactosylceramide and IL-2. Immunol Lett 2005.

(115) Gumperz JE, Roy C, Makowska A, Lum D, Sugita M, Podrebarac T et al. Murine CD1d-restricted T cell recognition of cellular lipids. Immunity 2000; 12(2):211-221.

(116) Lantz O, Bendelac A. An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans. J Exp Med 1994; 180(3):1097-1106.

(117) Behar SM, Podrebarac TA, Roy CJ, Wang CR, Brenner MB. Diverse TCRs recognize murine CD1. J Immunol 1999; 162(1):161-167.

(118) Crowe NY, Coquet JM, Berzins SP, Kyparissoudis K, Keating R, Pellicci DG et al. Differential antitumor immunity mediated by NKT cell subsets in vivo. J Exp Med 2005; 202(9):1279-1288.

(119) Shin T, Nakayama T, Akutsu Y, Motohashi S, Shibata Y, Harada M et al. Inhibition of tumor metastasis by adoptive transfer of IL-12-activated Valpha14 NKT cells. Int J Cancer 2001; 91(4):523-528.

(120) Gilboa E. DC-based cancer vaccines. J Clin Invest 2007; 117(5):1195-1203.

(121) June CH. Principles of adoptive T cell cancer therapy. J Clin Invest 2007; 117(5):1204-1212.

(122) Xue SA, Stauss HJ. Enhancing immune responses for cancer therapy. Cell Mol Immunol 2007; 4(3):173-184.

(123) Hodi FS, Mihm MC, Soiffer RJ, Haluska FG, Butler M, Seiden MV et al. Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients. Proc Natl Acad Sci U S A 2003; 100(8):4712-4717.

(124) Ribas A, Camacho LH, Lopez-Berestein G, Pavlov D, Bulanhagui CA, Millham R et al. Antitumor activity in melanoma and anti-self responses in a phase I trial with the anti-cytotoxic T lymphocyte-associated antigen 4 monoclonal antibody CP-675,206. J Clin Oncol 2005; 23(35):8968-8977.

(125) Sanderson K, Scotland R, Lee P, Liu D, Groshen S, Snively J et al. Autoimmunity in a phase I trial of a fully human anti-cytotoxic T-lymphocyte antigen-4 monoclonal antibody with multiple melanoma peptides and Montanide ISA 51 for patients with resected stages III and IV melanoma. J Clin Oncol 2005; 23(4):741-750.

(126) Phan GQ, Yang JC, Sherry RM, Hwu P, Topalian SL, Schwartzentruber DJ et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proc Natl Acad Sci U S A 2003; 100(14):8372-8377.

(127) Sutmuller RP, Morgan ME, Netea MG, Grauer O, Adema GJ. Toll-like receptors on regulatory T cells: expanding immune regulation. Trends Immunol 2006; 27(8):387-393.

(128) Liu H, Komai-Koma M, Xu D, Liew FY. Toll-like receptor 2 signaling modulates the functions of CD4+ CD25+ regulatory T cells. Proc Natl Acad Sci U S A 2006; 103(18):7048-7053.

Table I: Immunologic studies on iNKT cells in cancer patients

	n	peripheral blood iNKT levels	related to disease stage	iNKT cells in tumor infiltrate	relation to clinical outcome	peripheral blood iNKT IFN-γ production	proliferative response to αGalCer	cytotoxic against tumor [XI]
melanoma (Kawano TI, 1999) [31]	**13**	↓	?	?	?	?	↔	**yes**
prostate cancer (Tahir SMI, 2001) [32]	**6**	↓	?	?	?	↓ [VIII]	↓ [X]	?
lung cancer (Motohashi S, 2002) [33]	**60**	↓	**no**	**yes**	?	↔ [IX]	?	?
lung cancer (Konishi J, 2004) [34]	**55**	↔	**no**	?	?	?	↓	?
myeloma (Dhodapkar MV, 2003) [35]	**23**	↔	n/a	**yes**	**yes [V]**	↓	↔	**yes**
glioma (Dhodapkar KM, 2004) [36]	**9**	↔	n/a	?	?	↔	↔	**yes**
neuroblastoma (Metelitsa LS, 2004) [37]	**8 (blood) 98 (tumor)**	↔	n/a	**yes**	**yes [VI]**	?	?	?
various carcinomas (Crough T, 2004) [40]	**109**	↔ [I] ; ↓ [II]	n/a [I] ; ? [II]	n/a [I] ; ? [II]	n/a [I] ; ? [II]	n/a [I] ; ? [II]	n/a [I] ; ? [II]	n/a [I] ; ? [II]
various carcinomas (Molling JW, 2005) [41]	**120**	↓	**no [III]**	?	?	↔	?	?
various carcinomas (Yanagisawa K, 2005) [42]	**21**	↓	n/a	?	?	?	↓ [X]	?
head and neck squamous cell carcinoma (Molling JW, 2007) [57]	**47**	↓	?	?	**yes [VII]**	?	?	?
colorectal cancer (Tachibana T, 2005) [74]	**103**	?	n/a	**yes [III]**	**yes [VI]**	?	?	?

Overview of results from various studies on the number and function of circulating invariant natural killer T (iNKT) cells in cancer patients , their capacity to infiltrate tumors, or their relation to prognosis.
I: colorectal, renal cell and other cancers; II: breast cancer and melanoma; III: Not restored upon tumor de-bulking (18 weeks); IV: Vα24$^+$ T cells, assumed to be invariant natural killer T (iNKT) cells; V: IFN-γ secretion by iNKT in peripheral blood or tumor bed; VI: Size of tumor infiltrating iNKT cell pool; VII: Size of peripheral blood iNKT cell pool; VIII: after *in vitro* expansion using α-Galactosylceramide (αGalCer) loaded autologous peripheral blood mononuclear cells (PBMC) as antigen presenting cells (APC); IX:Detection of messenger RNA, not protein; X: Autologous αGalCer loaded PBMC used as APC, not monocyte derived dendritic cells; XI: upon *in vitro* expansion; ↓ , ↔ : reduced, unaffected compared to healthy controls respectively; ?: unknown (analysis was not performed); n/a: not applicable (no reduction in iNKT cells)

Table II: Clinical phase I studies regarding activation of peripheral blood iNKT cells in cancer patients

	therapeutic approach	n	clinical responses (when observed)	serum cytokines after therapy	effects on circulating iNKT cells after therapy	effects on other circulating cells after therapy
solid tumors (Giaccone G, 2002) [98]	i.v. αGalCer	24	stable disease (in 7 pt; 83-216 days)	elevated IL-12, IFN-γ (in 1/10 pt tested) TNF-α and GM-CSF (in 5/ 21 pt tested)	decline (in all pt)	transient reduction in NK cell number + cytotoxicity (in all pt tested)
metastatic malignancy (Okai M, 2002) [104]	i.v. immature αGalCer pulsed moDC	4	?	?	decline, followed by mild increase (in all pt)	?
metastatic carcinoma (Nieda M, 2004) [105]	i.v. immature αGalCer pulsed moDC	12	decreased serum tumor markers (in 2 pt; 4-12 months), tumor necrosis (in 1 pt), decreased serum liver enzymes (in 2 pt with hepatic metastases)	elevated IL-12 (in all pt) and IFN-γ (in 6/ 9 patients tested)	decline, followed by mild increase (in all pt)	T + NK activation and increased NK cytotoxicity (in 5/11 pt tested)
myeloma and carcinoma (Chang DH, 2005) [107]	i.v mature αGalCer pulsed moDC	3 and 2	decreased serum tumor markers (in 3 pt; 9-10 months) and stable disease (in 1 pt; 8 months)	elevated IL-12p40, MIP-1β and IP-10 (in all pt tested)	> 100 fold increase at peak (in all pt)	expansion of CMV specific $CD8^+$ T cells (in 3/3 pt tested)
advanced lung cancer [i] (Ishikawa A, 2005) [108]	i.v. autologous PBMC enriched for moDC pulsed with αGalCer	12	stable disease (in 3/9 pt tested; 23-26 weeks)	?	> 20 fold increase at peak (in 3/12 pt) and increase in iNKT IFN-γ mRNA (tested in 1 pt)	?
non small cell lung cancer [II] (Motohashi S, 2006) [109]	i.v. autologous PBMC enriched for iNKT cells	6	stable disease (in 4/6 pt; up to 12 moths)	?	mild increase (in 3/6 pt) and elevated IFN-γ in response to αGalCer in vitro (in 5/5 pt tested)	mild increase in NK cells (in 1/4 pt tested)

Overview of the clinical phase I studies conducted regarding activation of peripheral blood invariant natural killer T (iNKT) cells in cancer patients. Patients were treated with αGalCer, monocyte derived dendritic cells (moDC) or *ex vivo* activated iNKT cells. No adverse events were recorded in any of these studies. I: The method of DC preparation does not exclude the presence of *ex vivo* activated iNKT cells at time of injection; II: preparations contain large amounts of $CD3^+$ $Va24^-$ T cells and NK cells; ?: unknown (parameter not tested; pt: patients

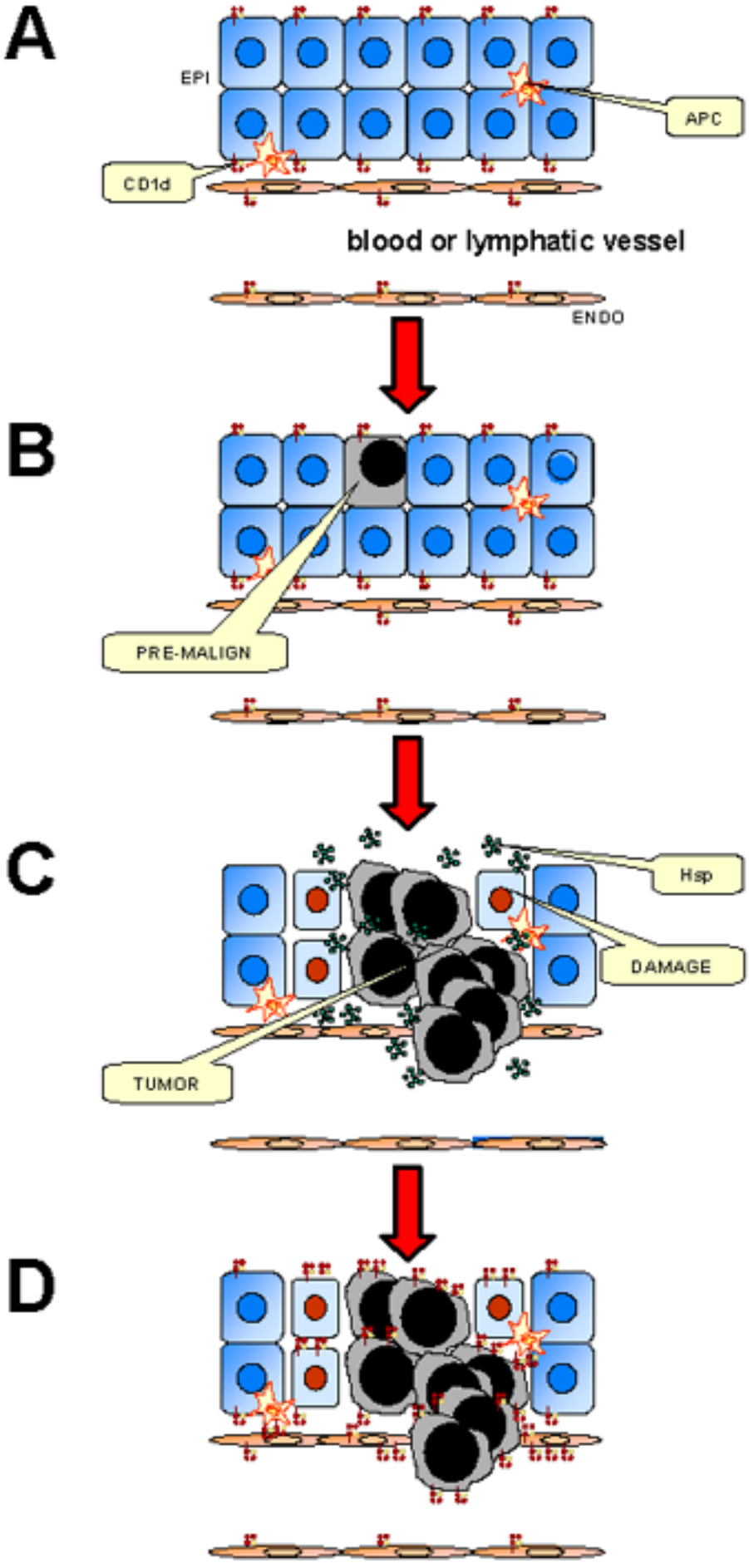
A
EPI
APC
CD1d
blood or lymphatic vessel
ENDO
B
PRE-MALIGN
C
Hsp
DAMAGE
TUMOR
D

Figure 1

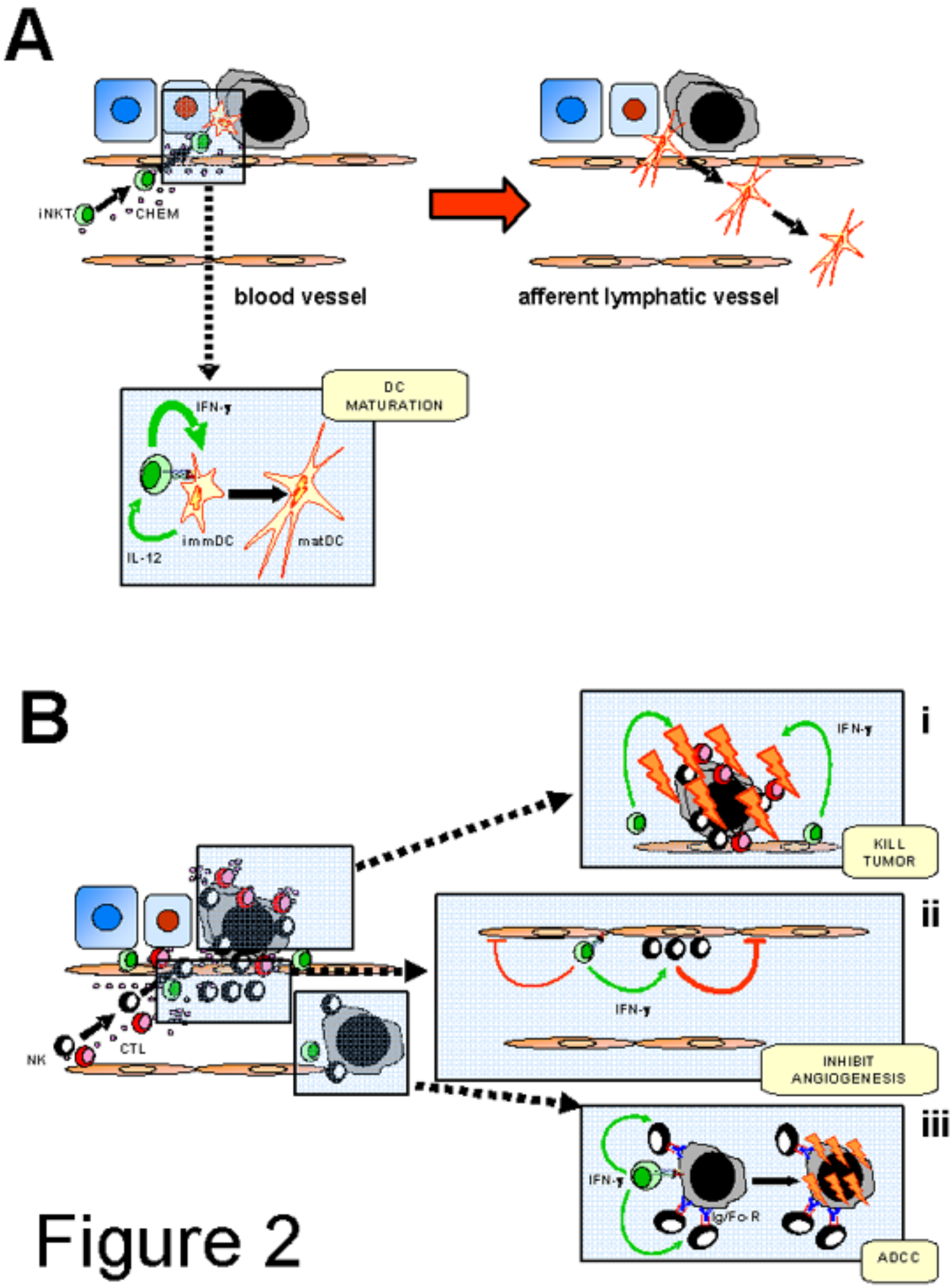
A
iNKT
CHEM
blood vessel
afferent lymphatic vessel
DC MATURATION
IFN-γ
immDC
matDC
IL-12
B
i
IFN-γ
KILL TUMOR
ii
IFN-γ
INHIBIT ANGIOGENESIS
iii
IFN-γ
Ig/Fc R
ADCC
NK
CTL

Figure 2